Sonolysis of Methylcob(III)alamin: Sequential absorption spectra of aqueous CH_3-Cbl^{III} as a function of anaerobic sonolysis (pH 7.38, 100 mM Hepes, saturating Ar) are shown in Figure 3A. The absorbance at 340, 374, and 520 nm decreases linearly as a function of sonolysis time and the absorbance at 316 and 420 nm increases linearly, thereby indicating the reaction is zero order in substrate concentration. The isosbestic points at 336, 390, and 585 nm are in agreement with those obtained through anaerobic photolysis of CH_3-Cbl^{III}. Under the conditions of sonolysis, an additional isosbestic point occurs at 476 nm, rather than at 486 nm, as typically observed in the course of photolysis. This slight shift in the isosbestic point is caused by a minor product that has an absorbance maximum near 490 nm. This may be cob(I)alamin that has a sufficient lifetime ($t_{½}$ = 22 min at pH 6) to be observed spectrophotometrically. The absorption band at 374 nm is characteristic of a C-Co bond, and its disappearance unambiguously indicates displacement of the axial carbon ligand.

Under aerobic conditions, molecular oxygen scavenges H• and prevents the reduction of CH_3-Cbl^{III} *via* the equation

$$O_2 + H\bullet \longrightarrow \bullet OOH$$

In the absence of an organic buffer with abstractable hydrogen atoms, reaction via HO• remains to be a viable process.

Figure 3B shows the change in absorbance spectra following aerobic sonolysis in the absence of organic buffer. The decrease in absorbance at 340 and 374 nm is linear with increasing sonolysis time indicating the reaction is zero order in substrate concentration, but the unexpected increase in absorbance at 520 nm indicates the stable product of cobalamin sonolysis is not hydroxocob(III)alamin, as would be expected if molecular oxygen were to reoxidize cob(II)alamin to cob(III)alamin. Aerobic photolysis under the same conditions shows the expected decrease in absorbance at 374 nm but no change at 520 nm. This difference suggests that HO• is able to displace the alkyl ligand from Co^{III}, but other HO• reactions also occur (perhaps through the secondary products HOO• and $\bullet O_2^-$) to oxidize the corrin ring. Similar absorbance spectra are obtained from sonolysis of an aerated aqueous solution containing 100 mM phosphate buffer.

A similar result is seen in the reaction of CH_3-Cbl^{III} with H• and HO• when these radical species are generated by pulse radiolysis (Blackburn et al., 1972). Reducing species H• reacts to produce the same spectral changes as shown in Figure 3A. Multiple oxidizing species (HOO•

and $\bullet O_2^-$) can react with CH_3-Cbl^{III} to cleave the Co-C bond, but these species also lead to the irreversible degradation of the corrin ring, as evidenced by the spectral changes similar to those seen in Figure 3B. A precedent for irreversible oxidation of the corrin ring exists in the photooxygenolysis of alkylcobalamins by singlet oxygen (Krautler and Stepanek, 1985).

Aerobic sonolysis of solutions containing 100 mM Hepes or 100 mM *t*-butyl alcohol produces no change in the absorption spectra over comparable time. This is because molecular oxygen quenches the H• reaction pathway, and *t*-butyl alcohol quenches the HO• reaction pathway. Although Hepes has not previously been reported to be a scavenger of HO•, many reports indicate that organic solute molecules such as formate, can inhibit the reaction of HO• (Weissler, 1962). The absence of any spectral changes under these conditions suggests that direct sonolysis of the Co-C bond is not an important reaction pathway.

EXAMPLE 3

Biological Testing Against NCI Human Tumor Cell Lines

The efficacy of the bioconjugates is tested against tumor cell lines using existing protocols for assessing the effectiveness of targeted coenzyme B_{12} antineoplastic agent-containing bioconjugates. Representative cell lines tested include HCT 116 (human colon tumor), A549 (human lung), ACHN (human kidney), MCF7 (human breast), human prostate, SK5-mel (human melanoma), KB (human nasopharyngeal), CCRF-CEM (human T-cell leukemia), HL-60 (human promyelocytic leukemia), RD-995 (mouse fibrosarcoma), B-16 (mouse melanoma) and Meth-A (mouse carcinoma). Drug screening is carried out with a colorimetric cell viability assay in a 96-well plate.

Additionally, selected bioconjugates are radiolabeled with ^{14}C or ^{3}H to assess the level of uptake by human tumor cells. As noted above, the prior art reports that some tumor and leukemia cells produce high levels of B_{12} binding proteins in the serum and sequester B_{12} in high concentrations of up to 50 fold.

Tumor Cell Line Testing Protocol: The drug bioconjugates, synthesized under procedures described herein or adaptions thereof, are diluted over 5 orders of magnitude (approximately 0.005 to 50 μg/mL). Four hours after seeding of the cell in the plate, the cells are treated with the appropriate drug dissolved in isotonic buffered solution. In control experiments, without photolysis triggered drug release, the drug is left on the cells for three

days, as in the normal basic cancer screen. In the wells for triggered drug release, the laser output is focused on selected wells for varying times and with varying intensity. Alternatively, a matrix of light-emitting diodes (LEDs) is used. The cells are incubated under standard mammalian tissue culture conditions under the proper CO_2 balanced atmosphere. After three days, the cells are re-fed and the colorimetric dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is added. The reduction of the MTT to purple formazan product is quantified in a 96 well plate reader. The concentration of the purple formazan dye is correlated with the number of viable cells. The reduction in cell survival at a given dose rate and photolysis exposure give a quantitative estimation of cell death and drug delivery effectiveness. Care is taken not to expose portions of the plate to photolysis conditions through adventitious spillover of radiation. This is accomplished by using 96 well plates with an opaque mask (Fisher cat# 07-200-565) for photolysis.

Uptake of Drug-B_{12} and Drug-Co[SALEN] Bioconjugates: The uptake of drug-B_{12} and drug-Co[SALEN] bioconjugates by cultured tumor cells is monitored by radiolabeling the drug or cobalamin during synthesis. ^{3}H-Labeled 5-fluorouracil, methotrexate and chlorambucil are purchased from DuPont/NEN (New England Nuclear). These drug bioconjugates, as well as ^{14}C-labeled methylcob(III)alamin (synthesized from Cob(I)alamin and $^{14}CH_3I$) provide an indication of receptor-mediated uptake by the various tumor cell lines. In this study, the cells are exposed to the radiolabeled drug as described in the preceding section, except no MTT is added at the end of the three-day incubation period. Since all of the cell lines except the leukemia cells grow while attached to the bottom of the microtiter plate well, the growth medium is aspirated to remove the unincorporated radiolabeled drug, followed by several washes with fresh medium. The labeled cells are detached from the bottom of the wells and the radioactivity quantified by scintillation counting. Growth of non-attached leukemia cell takes place in round-bottom microtiter plates such that centrifugation sediments the cells and allows washing with fresh growth medium before solubilizing the cells and quantifying the incorporated radiolabeled drug by scintillation counting.